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# CELL ADHESION TO CRYSTAL SURFACES: A MODEL FOR INITIAL STAGES IN THE ATTACHMENT OF CELLS TO SOLID SUBSTRATES

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## Abstract

This study addresses the mechanism of the chirally-restricted, RGD-independent adhesion of A6 epithelial cells to the {011} faces of calcium (R,R)-tartrate tetrahydrate crystals. The extensive and rapid adhesion of the cells to these surfaces, in the presence or absence of serum proteins, is distinctly different from the extracellular matrix-mediated adhesion to conventional tissue culture surfaces or to the {101} faces of the same crystals. The differences are manifested by insensitivity to ATP depletion, to disruption of microfilaments and microtubules and even to formaldehyde fixation of the cells. Furthermore, trypsin pretreatment does not affect cell attachment to the {011} faces, nor does trypsin post-treatment cause cell detachment from the crystals. We also noticed that the rapid adhesion to the crystal surface bears several lines of similarity to the early temporal stages in cell adhesion to regular tissue culture surfaces. Based on these observations and additional theoretical considerations, it is proposed that the molecular interactions responsible for the cell adhesion to the {011} surfaces may serve as models for an early "engagement" stage in cell adhesion which precedes, and may be essential for, the formation of stable and long-term contacts.

**Key Words:** Cell adhesion, crystal, epithelial cells, extracellular matrix, receptor-mediated adhesion.

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## Introduction

Cell adhesion to appropriate extracellular solid surfaces affects a large variety of processes in eukaryotic cells, including growth, migration and differentiation (Folkman and Moscona, 1978; Vasiliev and Gelfand, 1981; Sims *et al.*, 1992; Wang, *et al.*, 1993). It thus has a central role in many fundamental biological processes in multicellular organisms, including growth regulation, embryonic morphogenesis, organogenesis, wound healing, and malignant transformation (Geiger *et al.*, 1987, 1990; Burridge *et al.*, 1988). These features are manifested by cells plated on different physiological or artificial substrates and depend on the molecular structure of the substrate, the presence of the corresponding adhesion molecules at the cell surface, and the consequent transduction of transmembrane contact-induced signals (Ben-Ze'ev, 1991; Juliano and Haskill, 1993; Ruoslahti and Reed, 1994).

Cell adhesion has been shown to involve several distinct sequential and interdependent steps, including establishment of initial cell-substrate contacts, attachment to the solid surface and finally spreading on it (Grinnell, 1978; Grinnell and Hays, 1978; Bongrand *et al.*, 1982; Duval *et al.*, 1988). The later stages of the adhesive process involve interaction of transmembrane receptors (mostly of the integrin family) with specific epitopes on the extracellular matrix (ECM), and are cytoskeleton-dependent. The molecular basis for the initial stages in the adhesion to conventional substrates is still poorly defined, mainly due to the difficulty to experimentally "isolate" the initial binding from later temporal stages. An approach that might circumvent this difficulty, is the use of specific adhesive substrates that will induce the first temporal events in the adhesion cascade without evolving to subsequent stages.

Theoretical as well as experimental approaches have been developed to elucidate the molecular basis for substrate recognition and contact formation. Deterministic kinetic models were developed to predict the conditions under which adhesion occurs, taking into account the dynamics of attachment and detachment, and interactions with adhesion-promoting ECM components (Bell, 1978,

1981; Hammer and Lauffenburger, 1987; Cozens-Roberts *et al.*, 1990a; Cho *et al.*, 1993; Saterbak *et al.*, 1993). Receptor-mediated attachment was found to be mainly sensitive to changes in receptor density, heterogeneity, binding affinity, and cytoskeletal anchorage. The relative importance of these contributions may result in two extreme regimes of binding that have been defined as "rate controlled" and "affinity controlled" (Hammer and Lauffenburger, 1987).

Static and dynamic approaches were applied to the study of the molecular interactions underlying cell attachment and detachment. In the static approach, the number of adhering cells and their morphology were studied as a function of substrate properties (Grinnell *et al.*, 1977; Culp, 1983; Curtis *et al.*, 1983, 1986; van Wachem *et al.*, 1985, 1987; Lewandowska *et al*, 1989). Not surprisingly, cell adhesion, and especially its early phase, was profoundly affected by the chemical properties of the underlying substrate. The most adhesive substrates were those composed of hydrophobic polymers bearing hydrophilic and ionic groups. The free energy of the surface is a dominant factor in the initial stages of cellular attachment, either in the presence or in the absence of external serum proteins (Schakenraad *et al.*, 1989). Initial attachment was found to be more extensive in serum-free medium but these conditions induced only limited spreading (Curtis *et al.*, 1983). In contrast, a linear correlation was observed between cell spreading and ECM-protein adsorption on the surface (Horbett *et al.*, 1988). It was speculated that in serum-free medium and upon inhibition of protein synthesis, direct binding of cells to the substrate takes place (Curtis *et al.*, 1983).

In the dynamic approach, adherent cells were exposed to a steady laminar flow and cell detachment was measured as a function of the shear stress (Pratt *et al.*, 1989; Cozens-Roberts *et al.*, 1990b). Preadsorption of "adhesive proteins" on the surface increases cell spreading and decreases detachment under flow. This effect was apparent only following relatively long incubation times, suggesting that the adhesive mechanisms in the early and late stages are different (Schakenraad *et al.*, 1989; van Kooten *et al.*, 1992; Truskey and Proulx, 1993).

The use of crystals as adhesion substrates, as proposed in our previous studies (Hanein *et al.*, 1993, 1994), provides a unique possibility to define single phases in the adhesion process, due to the homogeneity of the surface, its regularity, and the knowledge available on its molecular structure. We have used cell adhesion to specific faces of calcium (R,R)-tartrate tetrahydrate crystals as a model for selective substrate recognition and attachment. We showed that cultured epithelial A6 cells attach massively to the {011} faces of calcium (R,R)-tartrate tetrahydrate crystals [but not to the {101} faces of the same crystals] within minutes after plating. This binding is apparently independent of exogenous proteins, and is not affected by addition of RGD-peptides or by the absence of serum in the medium. Furthermore this binding is stereospecific and does not occur on the mirror image {011} faces of calcium (S,S)-tartrate tetrahydrate crystals (Hanein *et al.*, 1994). It was proposed that the extensive stereospecific attachment to the {011} faces of calcium (R,R)-tartrate tetrahydrate crystals occurs via direct cooperative interactions between chiral cell surface molecules and exposed groups on the crystal surface.

It was further shown that attachment under these conditions is not followed by normal cell spreading and is incompatible with cell survival, possibly due to deprivation of viable adhesion signals (Hanein *et al.*, submitted). In the present study, we partially characterize the nature of the direct surface attachment and try to elucidate its possible relevance to cell adhesion in general, and to the mechanism of the very early steps of cell-substrate recognition and attachment, in particular.

## Materials and Methods

### Crystallization experiments

For each crystal system, optimal conditions for crystallization from aqueous solution were determined, ensuring that the crystals were well-formed, homogeneous, and reproducible with respect to morphology and size. All crystallization experiments were carried out at room temperature. Crystallization conditions for calcium (R,R)-tartrate tetrahydrate crystals were: 30 ml of 40 mM sodium hydrogen tartrate (Merck-Schuchardt, Darmstadt, Germany) was mixed with 30 ml of 43 mM $CaCl_2 \cdot 2H_2O$ (Merck-Schuchardt) at pH 6.5 and transferred to 35 mm cell culture dishes (Falcon, Becton Dickinson Labware, Plymouth, England). Typically, crystals of ~300-400 $\mu$m size formed within one day. Crystallization conditions for calcium (S,S)-tartrate tetrahydrate crystals were the same as for the (R,R) form except the use of (S,S) tartrate (Fluka Chemie AG, Buchs, Germany).

### Cell culture

A6 cells [Xenopus leavis kidney, epithelium-cell, American Type Cell Culture (ATCC, Bethesda, MD, USA; CCL 102) were cultured, at 28°C in Dulbecco's minimum essential medium (DMEM), supplemented with 10% fetal calf serum (Biolab Lab. Ltd., Jerusalem, Israel), in tissue culture dishes with or without crystals. When cells were plated on crystals, the experiments were performed on the same culture dishes in which the crystals were previously grown, still attached to the dish. To avoid crystal dissolution, all media, fixation, and washing solutions were saturated with respect to the

particular crystal used. As already described (Hanein *et al.*, 1993), the concentration of tartrate required to saturate the solutions does not, *per se*, affect cell growth and adhesion. Unless differently specified, the fixation was performed for 30 minutes with 3% paraformaldehyde.

### Plating of crystals on top of confluent A6 monolayers

Calcium (R,R)- or (S,S)-tartrate tetrahydrate crystals of 100-150 $\mu$m size were suspended in saturated complete medium, and plated on top of confluent cultures of A6 cells. Following 24 hours incubation, the dishes were rinsed twice and fixed. In the experiments selected for electron microscopy (EM) analysis, the cells were grown on 0.13 mm diameter glass cover slips (Chance Propper Ltd., Smethwick, Warley, England).

### Scanning electron microscopy (SEM)

Cells were fixed for 30 minutes with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2. The glass slides were rinsed twice and postfixed for 30 minutes with 1% osmium tetroxide in 0.1 M cacodylate buffer. The slides were rinsed (X2), dehydrated with ethanol and critical point dried with $CO_2$ (Autosamdri-810, Tousimis, Rockville, MD, USA). The glass slides were placed on carbon-coated stubs and sputter coated with gold. The specimens were examined in a JEOL JSM-6400 scanning electron microscope (JEOL Ltd., Tokyo, Japan) operated at accelerating voltages of 10 to 15 kV. The identification of crystal faces was performed as previously reported (Hanein *et al.*, 1993).

### ATP-depletion

Cells were treated with sodium azide, a cytochrome inhibitor, or with CCCP, an oxidative phosphorylation uncoupler. Prior to cell seeding, A6 cells were incubated for one hour with glucose-free DMEM or with DMEM containing 30 mM 2-deoxyglucose, 20 mM HEPES and 1% dialyzed fetal calf serum, in the presence of either 20 $\mu$M sodium azide or $10^{-5}$ M carbonyl cyanide m-chlorophenyl hydrazone (CCCP, Sigma, St. Louis, MO, USA; Bershadsky *et al.*, 1980; Glascott *et al.*, 1987). The treated cells were seeded in 35 mm Falcon dishes in the presence or absence of crystals in the same media. As a reference, treated cells were seeded in the same medium, to which 30 mM glucose (Sigma) was added immediately after seeding, or in complete medium. Following 60 minutes of incubation, the dishes were rinsed twice and observed under phase-contrast microscopy using a Zeiss IM35 inverted microscope (Oberkochen, Germany).

### Treatment with microfilament disrupting drugs

A6 cells, suspended in complete medium in siliconized (Sigmacote, Sigma) 13 x 100 mm borosilicate glass test tubes, were treated with 10 $\mu$M Cytochalasin D (CD; Sigma) for one hour prior to cell seeding. The treated cells were seeded in 35 mm Falcon dishes with or without crystals in complete medium, in the presence of the inhibitor. Following 60 minutes incubation, the dishes were rinsed twice and observed in a Zeiss inverted microscope.

### Treatment with microtubules disrupting drugs

Confluent cultures of A6 cells were incubated with 10 $\mu$M nocodazole for one hour (DeBrabander *et al.*, 1976; Middleton *et al.*, 1989; Breitfeld *et al.*, 1990). The treated cells were harvested with trypsin-versene and seeded in 35 mm Falcon dishes with or without the crystals in complete medium, in the presence of the inhibitor. Following 60 minutes incubation, the dishes were rinsed twice and examined with a Zeiss inverted microscope. Nocodazole (Sigma) was prepared from a stock solution of 5 mg/ml in dimethylsulfoxide (DMSO).

### Attachment of paraformaldehyde fixed cells to crystal surfaces

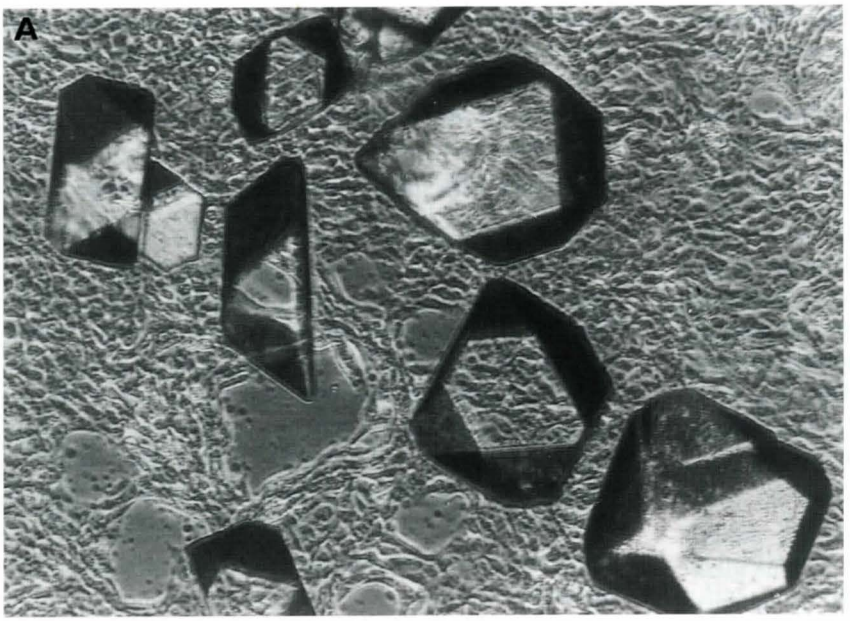
A6 cells were fixed for 20 minutes with 3% paraformaldehyde. The treated cells were rinsed twice and seeded in complete medium, in 35 mm Falcon dishes containing the crystals. Following 60 minutes incubation, the dishes were gently rinsed and examined with a Zeiss inverted microscope.

### Effect of cell harvesting technique on cell adhesion

Near-confluent cultures of A6 cells in 100 mm Falcon tissue culture dishes were rinsed twice in serum-free medium and detached either by 4 ml trypsin (0.25%)-EDTA (ethylenediaminetetraacetic acid, 0.02%; Biolab) or by 4 ml of 2 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA; Sigma) at 26°C (10 minutes). At the end of the treatment, the supernatant was mixed with 10 ml fresh complete medium. The cells were centrifuged at 1500 rpm for 5 minutes, resuspended in 1 ml serum-free medium, and $2.6 \times 10^4$ cells were replated in 55 mm Falcon tissue culture dishes. The cells were incubated for 15 minutes in either complete medium or serum-free medium. The non-attached cells were removed from the dish by gentle rinsing with fresh medium (either complete or serum-free, according to the specified experimental conditions). Ten microscope fields were photographed using a Zeiss inverted microscope, and the number of adhering cells was directly counted.

### Cell detachment assay

A6 cells were seeded in duplicate ($10^5$ cells in 35 mm Falcon dishes) either in complete medium or in serum-free medium, and incubated for different periods. The dishes were rinsed twice in serum-free medium and 2 ml of trypsin-EDTA solution was added. The detachment from the substrate was directly monitored with a Zeiss inverted microscope.



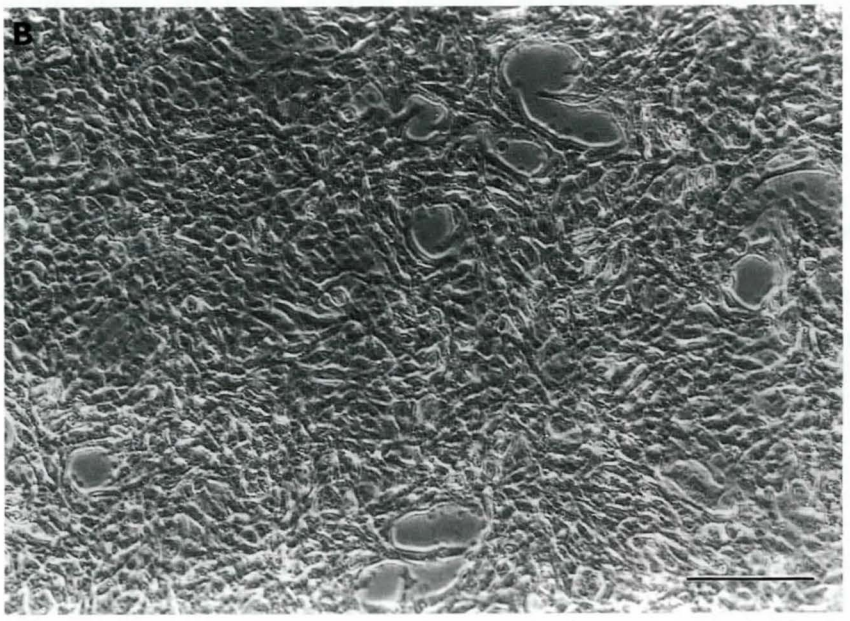



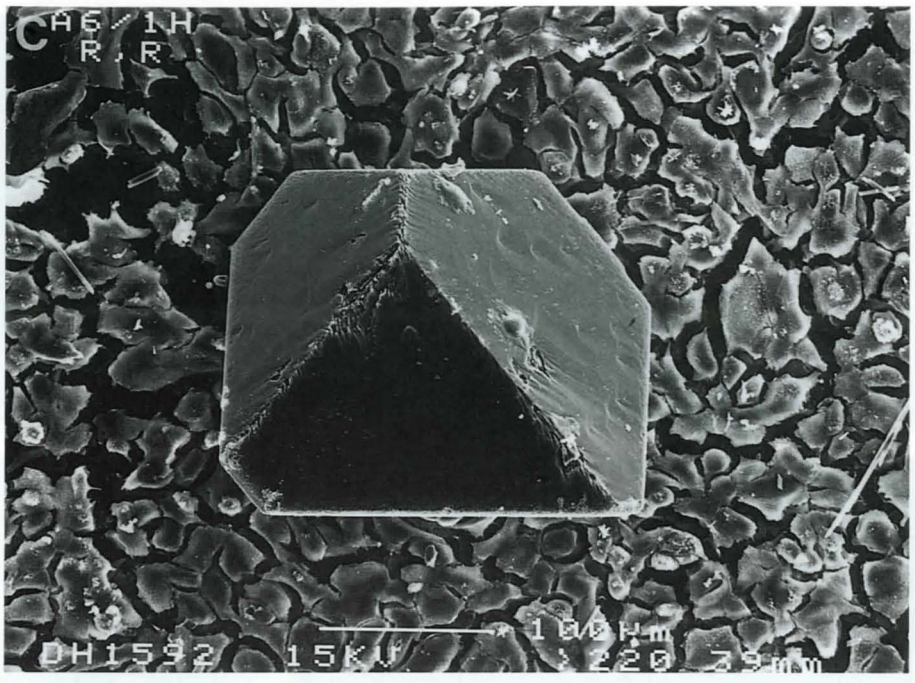

**Figure 1 (A and B at left; C and D on the facing page 201).** Phase-contrast photomicrographs of (A) calcium (R,R)-tartrate tetrahydrate crystals; and (B) calcium (S,S)-tartrate tetrahydrate crystals plated on top of a confluent culture of A6 cells. Bar = 50 $\mu$m (A and B are at same magnification).

## Results

### Selective binding of crystals to confluent cell monolayers

A6 cells in confluent monolayers are highly polarized cells, with an apical domain exposed to the culture medium and basolateral domain through which they adhere to the underlying matrix. To determine whether the membrane component(s) responsible for the stereoselective adhesion to the {011} crystal surface are also polarized, calcium (R,R)- or (S,S)-tartrate tetrahydrate crystals were plated on top of a confluent monolayer of A6 cells. To avoid excessive mechanical constraints on the cell monolayer, relatively small crystals were used (100-150 $\mu$m). The dishes containing the crystals and the cells were incubated for 24 hours, fixed and washed. A large number of (R,R)-crystals remained attached to the cell monolayer in the dishes (Figs. 1A and 1C), whereas only few crystals, if at all, could be detected in the dishes containing (S,S)-crystals (Figs. 1B and 1D) following mild washing.

SEM observations indicated that the (R,R)-crystals



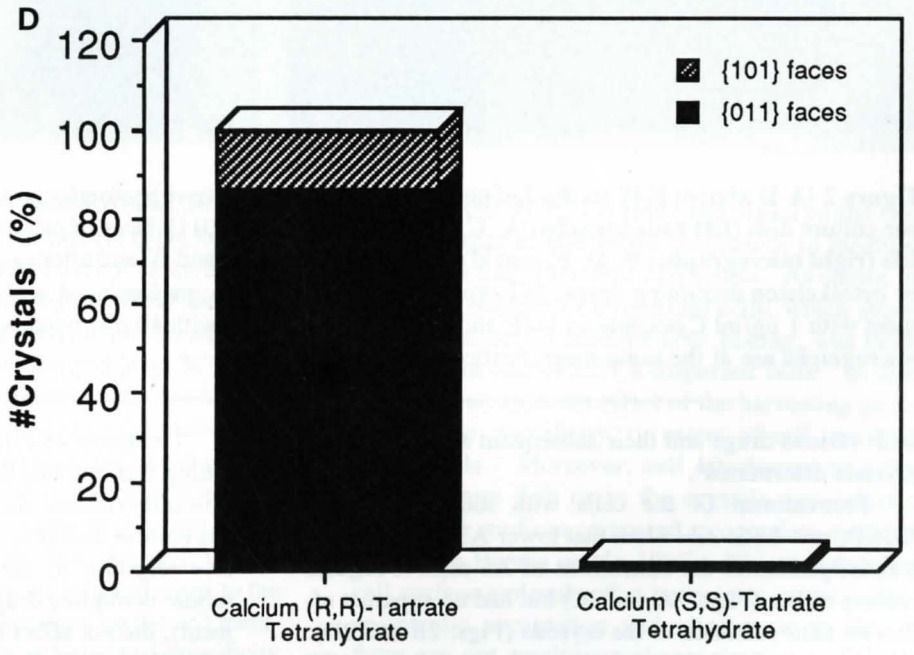




**Figure 1 (C and D at right; A and B on the facing page 200).** (C) Scanning electron micrograph of a crystal plated as in (A). Bar = 100 $\mu$m. (D) Histogram summarizing the selectivity of crystal attachment to the cell monolayers, showing that 80% of the (R,R)-enantiomeric crystal attach to the dorsal cell surfaces via the {011} faces. Practically no (S,S)-crystals are found attached to the cell monolayer.

attached to the dorsal cell surfaces preferentially via the {011} faces (Fig. 1D). The attached crystals did not perturb the extensive membrane foldings and ridges, which are typical of A6 cells, and were usually associated with the tips of the apical protrusions (Hanein *et al.*, submitted). Thus, the face-selective and enantioselective behavior is fully expressed at the apical surface of polarized A6 cells.

### The effect of cytoskeletal and energy metabolism inhibitors on cell-crystal interaction

Cell adhesion to conventional tissue culture surfaces or to the extracellular matrix requires an intact microfilament system and is sensitive to metabolic inhibitors (Bershadsky *et al.*, 1980). To determine whether the short-term adhesive interactions occurring at the {011} crystal surface show similar features, cells were treated

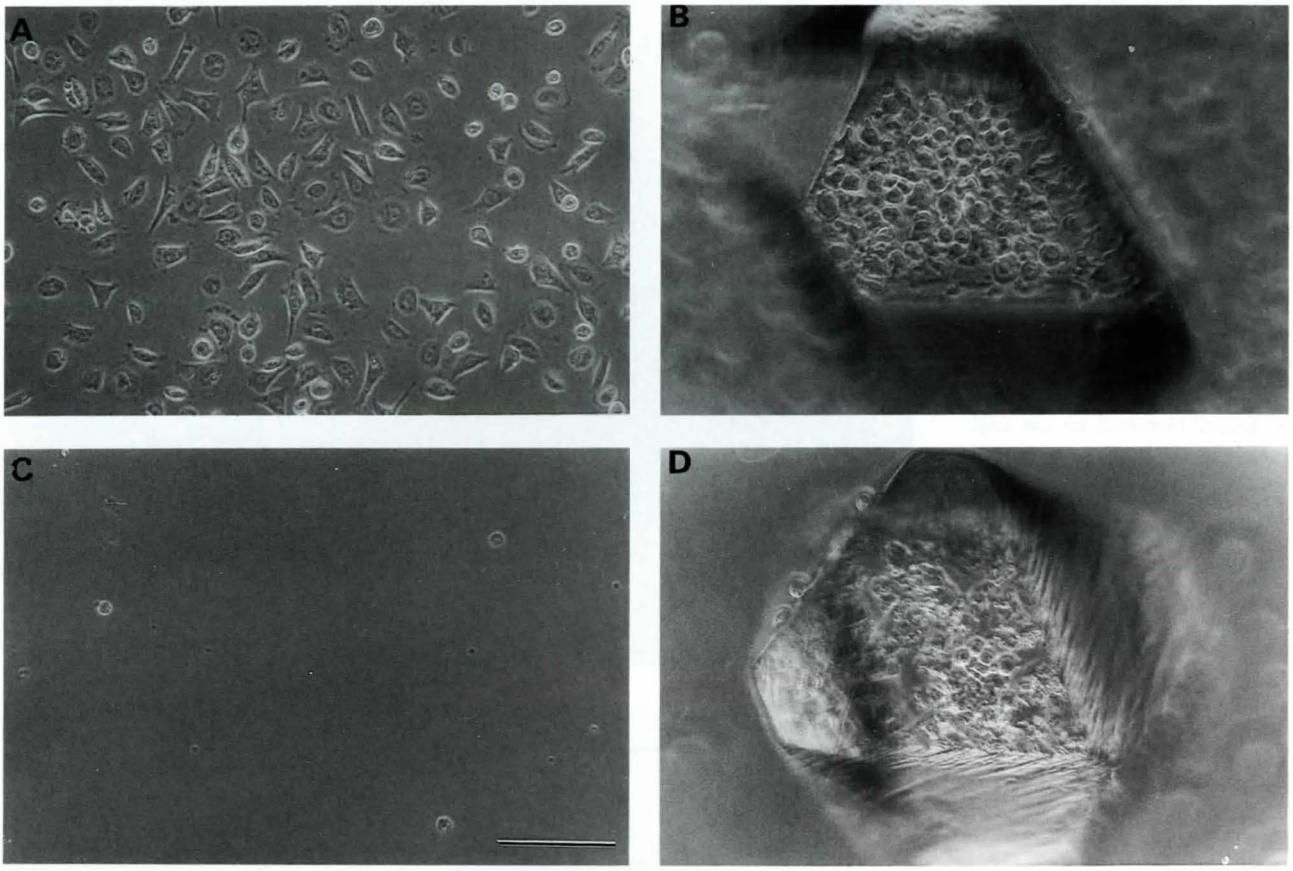



**Figure 2 (A-D above; E-H on the facing page 203).** Phase-contrast photomicrographs of A6 cells, attached to the tissue culture dish (**left micrographs: A, C, E, and G**) or to the {011} faces of calcium (R,R)-tartrate tetrahydrate crystals (**right micrographs: B, D, F, and H**), without treatment (**A** and **B**) and after pretreatment with metabolic inhibitors or cytoskeleton disrupting drugs, following 1 hour of incubation: pretreatment with 20 $\mu$M azide (**C** and **D**); pretreatment with 1 $\mu$g/ml Cytochalasin D (**E** and **F**); and pretreatment with 10 $\mu$M nocodazole (**G** and **H**). Bar = 50 $\mu$m (all micrographs are at the same magnification).

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with various drugs and their subsequent adhesion to the crystals determined[1].

Pretreatment of the cells with sodium azide or CCCP, metabolic inhibitors that lower ATP levels, substantially reduced the attachment of A6 cells to regular culture dishes (Figs. 2A and 2C) but had no apparent effect on their adhesion to the crystals (Figs. 2B and 2D). We can conclude that depletion of the cellular ATP pools and the consequent cellular responses do not affect the direct attachment to the crystals.

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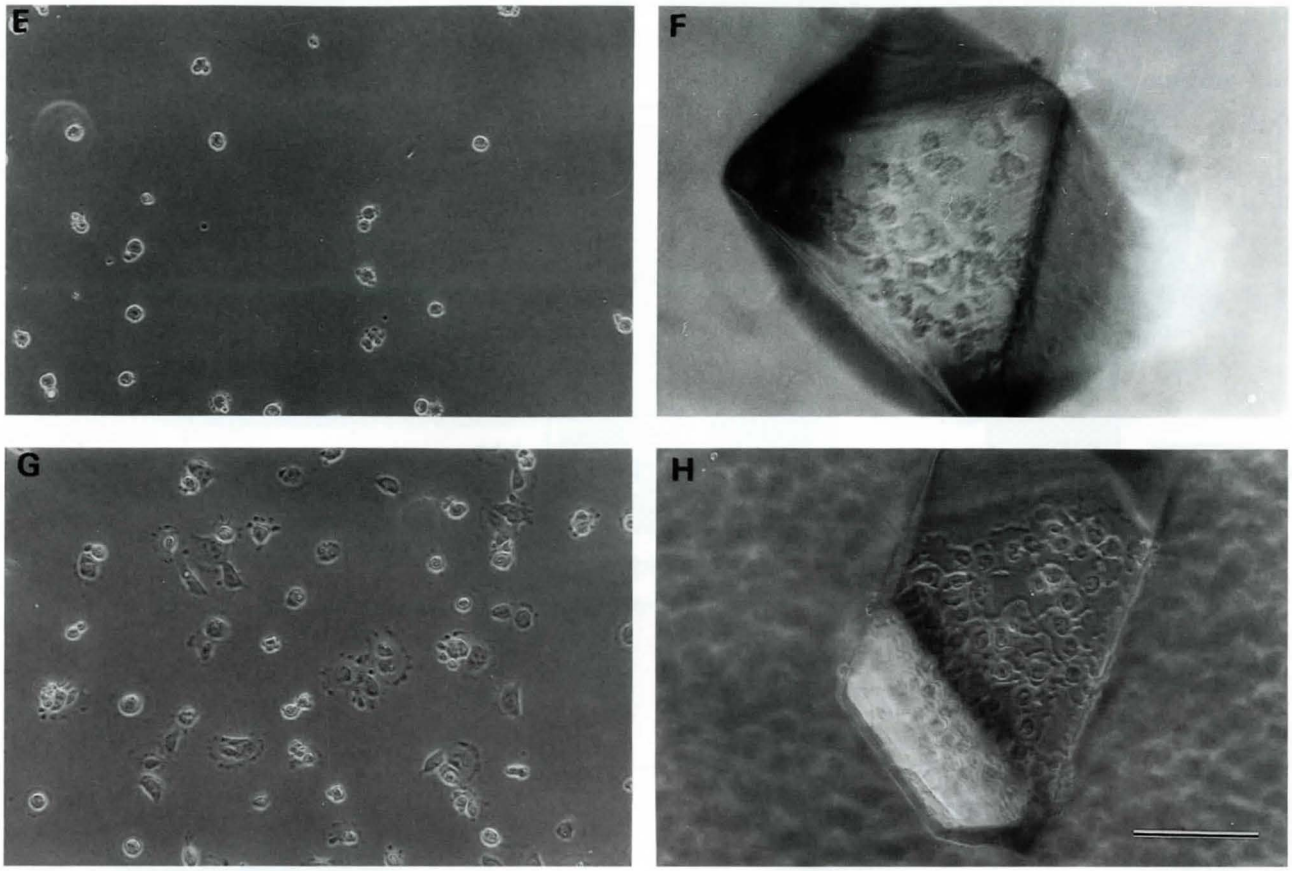
[1]**Note:** as direct, short-term cell attachment was observed specifically and exclusively on the {011} faces of calcium (R,R)-tartrate tetrahydrate crystals, the mention of crystal surfaces in all the following text will refer to these faces, unless differently specified.

Treatment of cells for 1 hour with Cytochalasin D, a drug that disrupts the actin microfilament system, significantly reduced the number of cells attached to the tissue culture dish (Fig. 2E) yet had no effect on the adhesive response to the crystals (Fig. 2F). The microtubule-disrupting drug nocodazole (10 $\mu$M, 1 hour treatment), did not affect the short-term adhesive response of A6 cells to both the crystals (Fig. 2H) and the culture plates (Fig. 2G), although it modified the morphology of the attached cells. In conclusion, it appears that cell adhesion to the crystal surface does not depend on either the actin- or microtubules-based cytoskeleton nor does it require normal energy metabolism.

### Adhesive behavior of paraformaldehyde fixed cells

The requirement for cell surface dynamics in the direct attachment to the crystals was challenged by chemically fixing the cells prior to their seeding on the




tartrate crystals. As shown in Figures 3A and 3B, within 60 minutes of incubation of normal A6 cells, both the crystals and the dish surface, respectively, were densely covered with cells. Gentle rinsing removed the fixed cells from the culture dish (Fig. 3D), while the adhesion to the crystals was apparently unchanged (Fig. 3C). The fixed cells display weaker attachment to the crystal than living cells, which may be attributed to the inability of their surface to dynamically rearrange and develop highly cooperative interactions. Notably, attachment remained selective to the {011} faces of the (R,R) crystals.

These observations indicate that the cell surface molecules that participate in the initial attachment to the crystal faces are permanently expressed on the cell membrane surface and their binding activity and specificity are not affected by formaldehyde fixation.

### Effect of proteolytic pretreatment on cell adhesion to the crystal surface

To determine whether surface proteins are involved in the attachment to the {011} faces of the crystals, we checked whether the cell harvesting procedure (mainly proteolytic pretreatment) affects either the rate, specificity or extent of the initial attachment of A6 cells to the crystals, and to the tissue culture dishes. Confluent cells were removed from tissue culture dishes either by trypsin-EDTA or by EGTA alone and plated on the two substrates, either in serum-free medium or in medium supplemented with 10% fetal calf serum. As shown in Figure 4, the number of trypsinized cells, which adhered to the dish within 15 minutes after plating, was three-fold higher than that of EGTA-dispersed cells. In contrast, there was no apparent effect of the harvesting procedure on the rate, specificity, or extent of cell attachment to the crystals. Moreover, cell attachment to either the tissue culture dish or to the crystals was enhanced in serum-free medium compared to complete medium (see Fig. 9a in Hanein *et al.*, 1993). This suggests that the cell surface molecules that participate in the initial stereo-specific recognition and attachment to the crystal faces are not sensitive to trypsinization or EGTA. In addition, exogenous proteins adsorbed on either the cells or the substrate might partially mask the specific epitopes that participate in the initial attachment process.

### Cell adhesion to the {011} crystal surfaces and initial attachment to tissue culture dishes are both largely trypsin insensitive

The time required for trypsin-EDTA induced detachment of A6 cells following 10-120 minutes adhesion to tissue culture dishes or to tartrate crystals was measured. The time required for complete detachment from

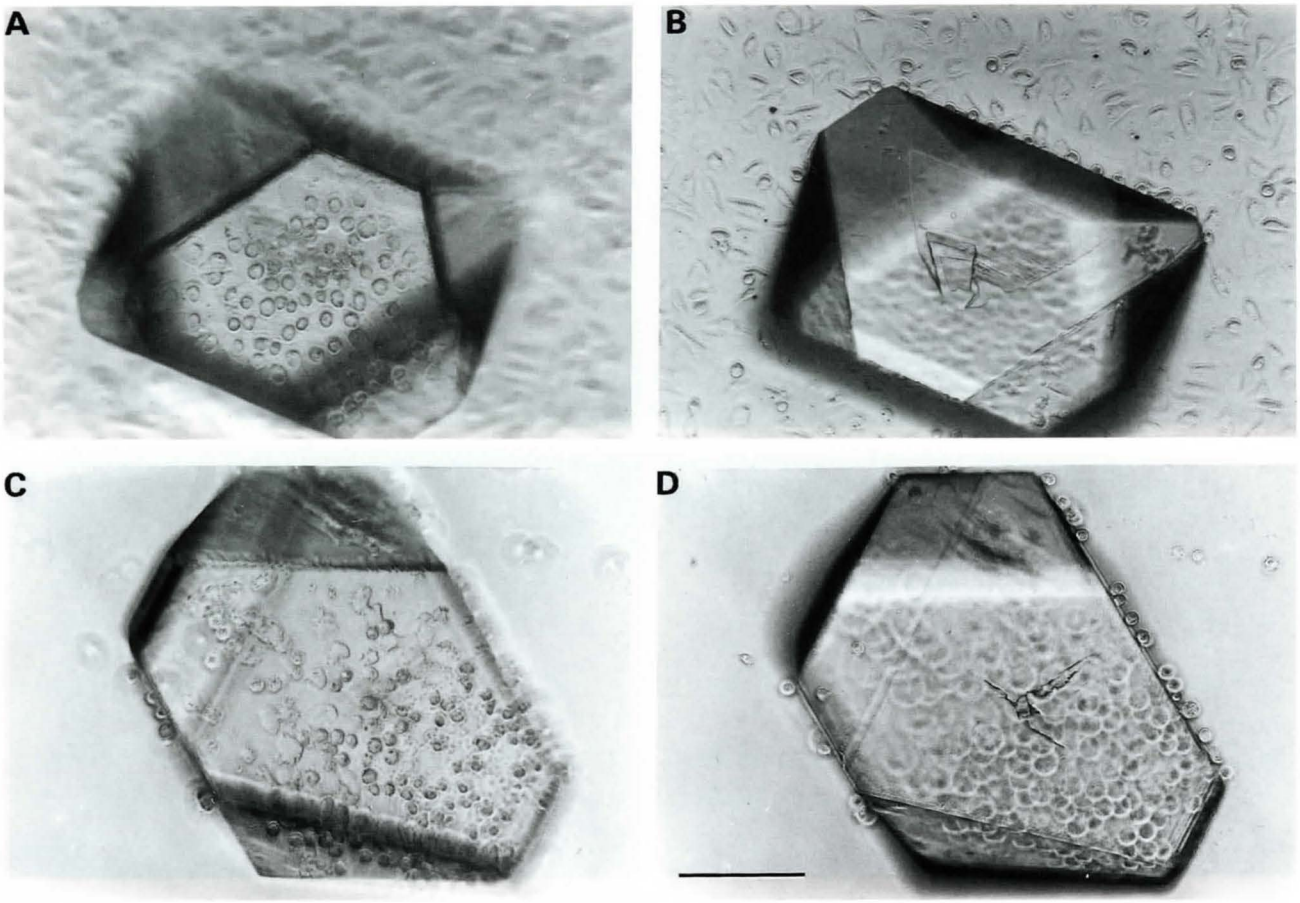



**Figure 3.** Phase-contrast photomicrographs taken on two focal planes at the same lateral location. The lower focal plane is focused on the surface of the dish, showing cells attached to the tissue culture dish (**right panel**). The higher focal plane is focused on the top (011) face of calcium (R,R)-tartrate tetrahydrate crystals, showing cells attached to this crystal face (**left panel**). Normal viable cells (**A** and **B**). Cells replated following 20 minutes fixation with 3% paraformaldehyde (**C** and **D**). Bar = 50 $\mu$m (the four micrographs are at the same magnification).

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the tissue culture dishes decreases with increasing pre-incubation time, reaching a plateau after 120 minutes of pre-incubation (Fig. 5). In contrast, cells attached to the crystal faces did not detach (up to 3 hours of treatment), regardless of the pre-incubation time. Interestingly, cell attached to the culture dish in serum free medium, remained trypsin-resistant for an entire hour after plating. Upon longer pre-incubation (approximately 2 hours), trypsin sensitivity became apparent, though cell detachment occurred at a slower rate compared to cells incubated in complete medium. Cells incubated in serum-free medium on culture dishes preconditioned with 10% fetal calf serum, showed the same time-dependence of detachment as cells incubated with complete medium, suggesting that the trypsin-sensitive elements are, at least partly, substrate-attached adhesive proteins.

## Discussion

The main objective of this study was to elucidate the molecular and cellular mechanisms underlying the direct, RGD-independent cell adhesion on the {011} faces of calcium (R,R)-tartrate tetrahydrate crystals (Hanein *et al.*, 1993, 1994). The main observations which provided the basis for the present study, may be summarized as follows: (a) A6 epithelial cells distinguish between the two face-types of the same crystals; they bind within minutes after plating to the {011} faces, whereas adhesion to the {101} faces occurs only after many hours in culture; (b) cell binding to the {011} faces is chirally restricted; it occurs only on the (R,R)-, and not on the (S,S)-, enantiomer and it is cell-type specific; (c) cell adhesion to the {011} faces leads to cell death while the

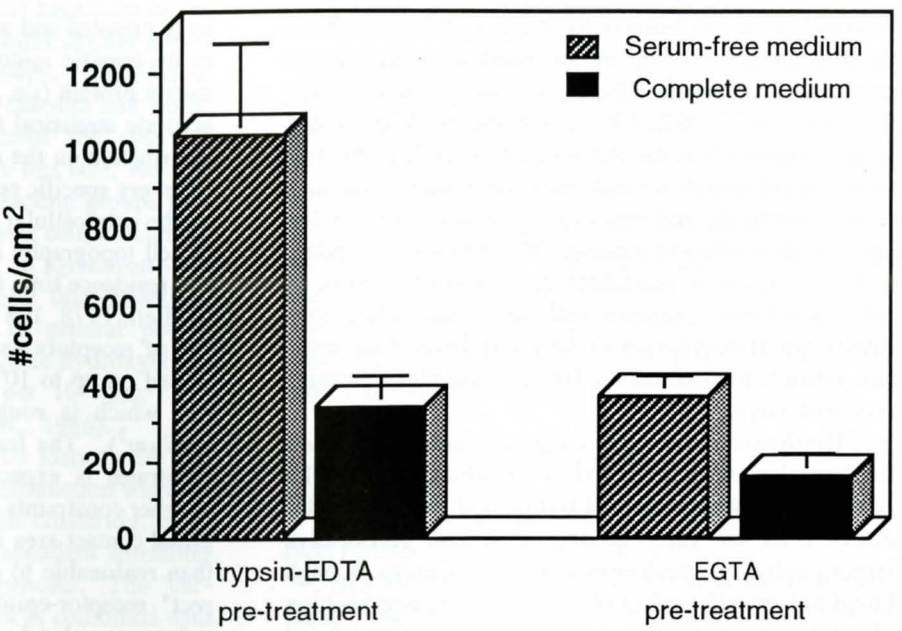


**Figure 4.** The effect of cell harvesting procedure and medium composition on the attachment of A6 cells to the tissue culture dish. Pretreatment before replating included application of 0.25% trypsin-0.02% EDTA (**left**) or 2 mM EGTA (**right**). Cells were replated in either serum-free medium or complete medium. Error bars represent standard deviations.

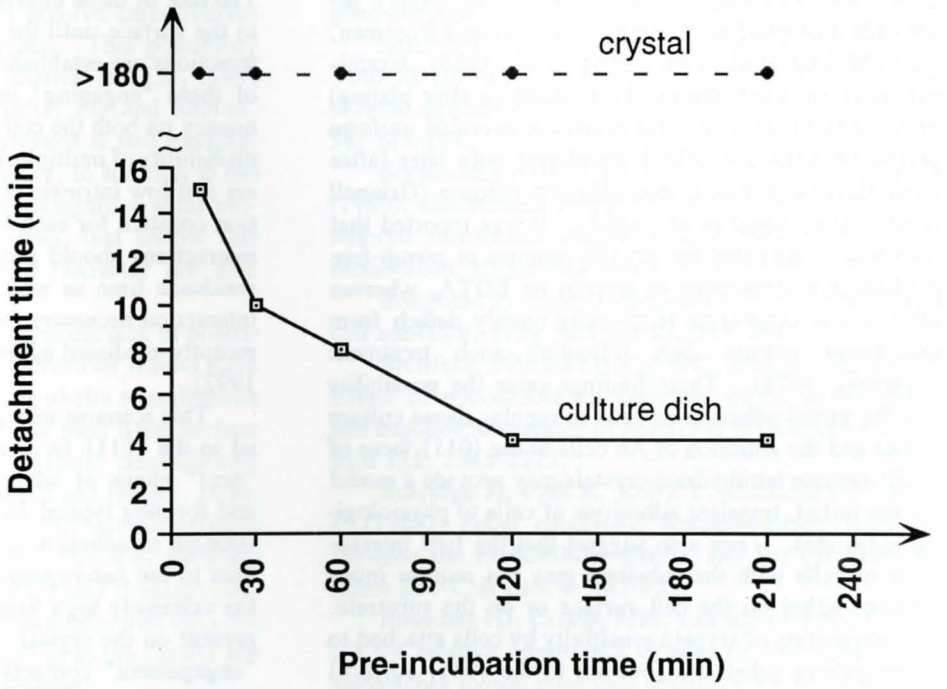


**Figure 5.** Time required for the detachment of A6 cells from the tissue culture dishes or the {011} faces of calcium (R,R)-tartrate tetrahydrate crystals, induced by trypsin. The detachment time is plotted as a function of the pre-incubation time in complete medium.

"slow adhesion" to the {101} faces supports cell survival and proliferation.

In this paper, we have characterized the short-term adhesion to the {011} surface and determined their possible relevance to "physiological" cell adhesion to complex surfaces, either natural or artificial. We show that unlike their adhesion to regular tissue culture substrates or even to the {101} surfaces of the same crystals, the attachment of A6 cells to the {011} surfaces does not require energy metabolism and is not affected by disruption of F-actin or microtubules or restrictions in membrane dynamics. In fact, rapid cell attachment to the crystals was not inhibited even by chemical fixation. Stereo-selective and enentio-selective cell-crystal interaction occurred at the dorsal membrane of fully spread and polarized cells. Finally, cell detachment from the crystal {011} surfaces is insensitive to trypsin, as is cell detachment from tissue culture dishes after short incubation, especially in the absence of serum in the medium.

The results of these experiments provide substantial

information on the binding mechanism, over and above the trivial negation of the involvement of certain components in the process. Based on our previous results (Hanein *et al.*, 1993, 1994), and the work presented here, it appears that the adhesion of A6 cells to the two face-types of tartrate crystals bear, each, close similarity to two temporally and molecularly distinct stages in the physiological adhesive process. We propose that binding to the {011} faces resembles the initial attachment of cells to adhesive surfaces such as tissue culture substrates, whereas adhesion to the {101} faces of the crystals appears to represent a later, extracellular matrix-mediated stage.

The distinction between temporal phases in adhesion has been described for a variety of cultured cells. The attachment and spreading of trypsinized Xenopus endothelial cells on glass occurs in several consecutive stages, including attachment to a solid substrate through broad and smooth contact sites, followed by organization of actin into stress fibers and extension of peripheral lamellae (Bereiter-Hahn *et al.*, 1990). The first phase, unlike the later stages, was shown to be largely independent of metabolic energy (Umbreit and Roseman, 1975; McClay *et al.*, 1981; Duval *et al.*, 1988). Examination of the early stages (15-30 minutes after plating) of cell attachment to a solid substrate, revealed uniform peripheral adhesions which developed only later (after more than 60 minutes) into adhesion plaques (Grinnell *et al.*, 1976; Segel *et al.*, 1983). It was reported that fibroblasts cultivated for 60-180 minutes in serum-free medium are insensitive to trypsin or EDTA, whereas after longer incubation these cells readily detach from the tissue culture dish following such treatment (Takeichi, 1971). Their findings raise the possibility that the initial adhesion of cells to regular tissue culture dishes and the adhesion of A6 cells to the {011} faces of (R,R)-tartrate tetrahydrate crystals may provide a model for the initial, transient adhesions of cells to physiological substrates. They also suggest that the first interactions of cells with the substrate may not require intact proteins either on the cell surface or on the substrate. The acquisition of trypsin sensitivity by cells attached to tissue culture substrates [but not to the {011} surface] occurs concomitantly with the spreading of the cells.

The physiological relevance of the two-stage adhesion model deserves an additional elaboration. We would like to suggest that the development of highly cooperative cytoskeleton-dependent extracellular matrix-integrin-adhesions (such as, focal contacts) depends on an earlier set of interactions which we define here as "engagement" interactions.

We argue that in the initial encounter of a cell, suspended in solution, with a solid substrate, the probability of a random "direct hit", juxtaposing an appropriately oriented and accessible receptor (such as, integrin) to its specific epitope (i.e., RGD) on an extracellular matrix protein (i.e., fibronectin), is extremely low. An accurate statistical treatment is difficult without precise information on the number and distribution of receptors for every specific cell, and of the corresponding epitopes on the extracellular matrix. Moreover, parameters such as cell topography and deformability, cell contact area, and residence time for encounter, must also be considered (Bell, 1978, 1981). In any case, the fractional coverage of receptors will be in the range of less than 0.1% (based on up to $10^6$ randomly distributed receptors per cell which is roughly equivalent to $5 \times 10^3$ receptors/$\mu m^2$). The fractional coverage of epitopes on the substrates is expected to be much lower, with even tougher constraints of orientation and accessibility. The initial contact area is thus very sparsely populated. It is than reasonable to assume that the probability of "correct" receptor-epitope encounters would be very low, unless preceded by transient but highly effective interactions between cell surface and substrate components. The task of these interactions is to keep the cell tethered to the surface until the appropriate receptor-epitope interactions are established. The expected characteristics of these "engaging" interactions should be: high frequency on both the cell and the substrate, to ensure high probability of multiple, essentially simultaneous encounters and low intrinsic affinity, to ensure a high dissociation constant for each bond. An array of such multiple interactions should provide the cell with the sufficient residence time as well as with the reversibility of the interaction necessary for the establishment of long-term receptor-mediated adhesive interactions (Busscher *et al.*, 1992).

This scenario may also explain why the cells attached to the {011} faces of tartrate fail to proceed to the "next" phase of adhesion acquiring RGD-dependence and forming typical focal contacts. It is likely that the strength of adhesion to the {011} faces is much greater than to the heterogeneous tissue culture surface due to the extremely high density of identical binding moieties present on the crystal. An excessive formation of such "engagement" contacts may lead to massive initial attachment that is too tight to allow further rearrangement and focal contact formation.

In contrast to conventional substrates, the distribution of groups on the crystal surfaces is fully determined. The section of the crystal unit cell on the {011} surface is of 100 $Å^2$, meaning that the density of units homogeneously repeated all along the substrate surface is of $10^6/\mu m^2$. This density of potentially interactive groups is clearly not reached on the heterogeneous, amorphous, conventional substrates. On the calcium tartrate tetrahydrate {011} faces are exposed rows of

tartrate molecules, emerging with their backbone to the surface, alternatively exposing the chiral carbon atoms with the hydroxyl or hydrogen groups. The {101} faces, exposing carboxylate groups, calcium and water in a different configuration, represent the opposite extreme of the engagement range. On this face, we suggest, the absence of appropriate interacting groups prevents engagement, resulting in delayed cell attachment (> 24 hours) (Hanein *et al.*, 1993). In agreement with this interpretation, it has been shown that substrates containing carboxylate groups are in general poorly adhesive, while substrates decorated with hydroxyls (up to a limit density of 2000 groups per $10^{-3}$ $\mu m^2$) are inducive to cell adhesion (Curtis *et al.*, 1986).

While these results do not directly identify the cell surface molecule(s) involved in the interaction with the {011} faces, they do suggest that their clustering, cytoskeletal anchorage or even energy dependent spreading are not essential for the attachment process. The "carbohydrate-like" nature of the {011} faces combined with the apparently passive, yet stereo-specific, attachment mechanism lead us to speculate that the initial recognition and attachment event might involve carbohydrate moieties present on the cell surface. This possibility was amply discussed in the past (Sharon and Lis, 1989; Lochner *et al.*, 1990; Elbein, 1991; Lis and Sharon, 1993), yet specific supporting evidence is still missing. It will be interesting to know whether, in addition to the short-range interactions governing recognition at the molecular level, long-range interactions, that endow this surface with an overall attracting character are also involved.

Another interesting observation is the lack of polarity in the cell-crystal interaction. Previous results have shown that polarized cells such as epithelia or endothelia preferentially adhere to the ECM via their basal surface and that the apical surface is largely non-adhesive (DiPasquale and Bell, 1974; Grinnell and Geiger, 1986; Schmidt *et al.*, 1993). The fact that tartrate crystals adhere to the apical surfaces of the cells with the same face-specificity [{011} versus {101}] and stereospecificity [(R,R) versus (S,S) crystals] indicates that the cell-surface molecules involved in binding to the crystal are exposed also at the apical compartment.

The use of crystals as adhesive surface models seems to have some unique advantages over conventional adhesive substrates, due to their highly uniform structures. It enables the distinction between individual temporal stages in adhesion processes. Furthermore, it allows the characterization of molecular and structural parameters that are directly involved in these distinct temporal stages. In the present study, this advantage was used to elucidate the molecular characteristics of the very first interactions between cell and substrate.

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## Discussion with Reviewers

**J.L. Brash**: The data showing no effect of harvesting methods on adhesion to the crystal should be shown.

**Authors**: The number of cells/unit area attached to the {011} faces of the crystals is the same, as judged by direct counting from light and electron micrographs. We preferred not to include the data in Figure 4 because they are not graphically compatible with the data reported in the figure. The order of magnitude is 1000 cells/mm$^2$ (see Fig. 6 in Hanein *et al.*, 1993), as opposed to 1000 cells/cm$^2$ on the tissue culture dishes.

**J.L. Brash**: In **Discussion**, you say: "In any case, the fractional coverage of receptors will be in the range of less than 0.1% (based on up to $10^6$ randomly distributed receptors per cell which is roughly equivalent to 5 x $10^3$ receptors/$\mu$m$^2$). The fractional coverage of epitopes on the substrates is expected to be much lower, with even tougher constraints of orientation and accessibility." Why is the epitope coverage on the substrate expected to be much lower than 5000/$\mu$m$^2$?

**Authors**: The maximum possible coverage, based on the area of fibronectin and a monolayer of molecules packed side by side, is roughly 600 molecules/$\mu$m$^2$, which is much lower than 5000/$\mu$m$^2$. However, even this is an unreasonably high limit because on physiological surfaces such a densely packed monolayer of ECM molecules (e.g., fibronectin) cannot be formed upon adsorption from serum. The maximum adsorption of fibronectin measured on treated polystyrene surfaces, based on data from Grinnell and Feld (1981), and Chilkoti *et al.* (1989), varies between 3-320 ng/cm$^2$, or 44-4400 molecules/$\mu$m$^2$. In those studies, it is not known whether the molecules are adsorbed side by side or in multilayered patches. Moreover, it is likely that at least part of the RGD epitopes on the surface-bound ECM molecules is not accessible to the surface receptors. It should be clear that fibronectin is just an example. The same principle should apply to other ECM molecules as well.

**J.L. Brash**: The main discussion of the paper is with regard to the temporal stages of adhesion, with attempts made to distinguish an initial or engagement phase from a later ligand-receptor phase. It is unclear what time scales the authors have in mind. Also how do the different times used in the experiments [1 hour for the experiments using cells treated with inhibitors etc., 24 hours for the adhesion of crystals to the cells (Fig. 1), and 15 minutes for the harvesting study] fit on this time scale?

**Authors**: Distinction must be made between the times used experimentally to measure adhesion, and the timescale of the "engagement" interactions. The latter is very short, of the order of Brownian motions in a liquid. However, the effect of the transient interactions persists well beyond this time scale and is manifested by long-term adhesion. Thus, the experimental time scale may be extended, provided no other mechanism of adhesion becomes operative in the same time scale. This is indeed the advantage of our experimental system: in regular adhesions, the maturation of the initial interactions into focal contacts is fast and continuous, whereas no maturation is observed on the crystal surfaces for many hours.

**J.L. Brash**: The **Discussion** (particularly paragraph 7) leaves the reader tantalized, but dangling. Terms like "different configuration", "appropriate interacting groups", are vague and unhelpful for understanding what is going on. With knowledge of the crystal surfaces as

advanced as the authors suggest, they should be able to be more specific.
**Authors**: We are well aware that all the knowledge we have on the crystal surface structure, however precise, is not sufficient to identify the interacting molecules on the cell surface. This is definitely a very important, and far from trivial, issue that has to be addressed. Prof. Brash must, however, concede that the comparison between the structures of the two crystal surfaces, correlated with the respective cell behavior, does provide information that would not be available from conventional substrates.

**J. Bereiter-Hahn**: The authors state: "Thus, the face-selective and enantio-selective behavior is fully expressed at the apical surface of polarized A6 cells." In polarized kidney epithelial cells, the apical surface can be expected to be endowed with a well developed apical fibrillar layer. This layer can be assumed to be under tension (developed by contractile elements spanning from opposite intercellular boundaries), therefore, this surface will be stiffer than other surfaces with less fibrillar support. The stiffness of the structure may counteract spreading of the contact area, and thus, decreasing the ability of the apex to adhere as strongly as do less differentiated cell areas. I would like to know the opinion of the authors concerning this interpretation.
**Authors**: The comment is correct and very insightful. The characteristic features of crystal attachment to the apical cell surface will be discussed more extensively in another manuscript (presently in preparation), where the transmission electron micrographs will exactly illustrate the point made by Prof. Bereiter-Hahn.

**A.L. Boskey**: Do all cell types show this pattern of adhesion to these crystals or does the pattern vary with cell type? Might such a survey provide some insight into which membrane components are involved?
**Authors**: In Hanein *et al.* (1994), we showed that the pattern of adhesion is cell type dependent. It indeed does differ for cell lines with different binding properties. We have not yet attempted to use this information to identify the membrane components involved.

**A.L. Boskey**: Were control for the nocodazole given the same amount of DMSO?
**Authors**: No.

**O. Johari**: Please provide more information about the Hanein *et al.* (submitted).
**Authors**: This paper "Hanein D, Sabanay H, Addadi L, Geiger B. Cell adhesion to crystal surfaces: (B) Adhesion induced physiological cell death." has been submitted to the Journal of Cell Biology.

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